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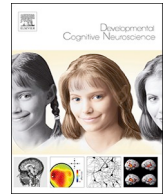
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# Adolescent pruning and stabilization of dendritic spines on cortical layer 5 pyramidal neurons do not depend on gonadal hormones

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## ABSTRACT

Pyramidal neurons in the neocortex receive a majority of their synapses on dendritic spines, whose growth, gain, and loss regulate the strength and identity of neural connections. Juvenile brains typically show higher spine density and turnover compared to adult brains, potentially enabling greater capacity for experience-dependent circuit ‘rewiring’. Although spine pruning and stabilization in frontal cortex overlap with pubertal milestones, it is unclear if gonadal hormones drive these processes. To address this question, we used hormone manipulations and *in vivo* 2-photon microscopy to test for a causal relationship between pubertal hormones and spine pruning and stabilization in layer 5 neurons in the frontal cortex of female mice. We found that spine density, gains, and losses decreased from P27 to P60 and that these measures were not affected by pre-pubertal hormone injections or ovariectomy. Further analyses of spine morphology after manipulation of gonadal hormones suggest that gonadal hormones may play a role in morphological maturation and dynamics. Our data help to segregate hormone-sensitive and hormone-insensitive maturational processes that occur simultaneously in dorsomedial frontal cortex. These data provide more specific insight into adolescent development and may have implications for understanding the neurodevelopmental effects of changes in pubertal timing in humans.

## 1. Introduction

As the site of most excitatory synapses and some inhibitory and modulatory synapses, dendritic spines are critical mediators of information processing by cortical pyramidal cells (DeFelipe and Farinas, 1992; Holtmaat and Svoboda, 2009; Spruston, 2008). Dendritic spines can be dynamic, with spines being both gained and lost during development (Holtmaat et al., 2005; Johnson et al., 2016a; Zuo et al., 2005a) and as a result of experience (Fu et al., 2012; Munoz-Cuevas et al., 2013; Xu et al., 2009; Yang et al., 2009). Although new and transient spines do not always contain synapses, spine gain and loss are thought to reflect the sampling of new potential synaptic partners and enable the remodeling of connectivity (Berry and Nedivi, 2017; Holtmaat et al., 2005; Trachtenberg et al., 2002; Villa et al., 2016; Zito et al., 2009), particularly in response to learning (Fu et al., 2012; Hayashi-Takagi et al., 2015; Lai et al., 2012; Munoz-Cuevas et al., 2013; Roberts et al., 2010; Xu et al., 2009; Yang et al., 2009). Changes in individual spine morphology are also known to occur with maturation of a new synapse and during development (Berry and Nedivi, 2017).

Spines in the frontal cortex, as well as other cortical regions, are

pruned during adolescence in a variety of species, including humans (Petanjek et al., 2011), rats (Koss et al., 2014), and mice (Holtmaat et al., 2005; Johnson et al., 2016a). *In vivo* imaging studies in mice using fluorescently labeled neurons have shown that spines are not only pruned, but also stabilized across adolescence: The fraction of total spines gained and lost per day declines during adolescence, leaving behind a more stable population as animals progress into adulthood (Holtmaat et al., 2005; Johnson et al., 2016a; Zuo et al., 2005a).

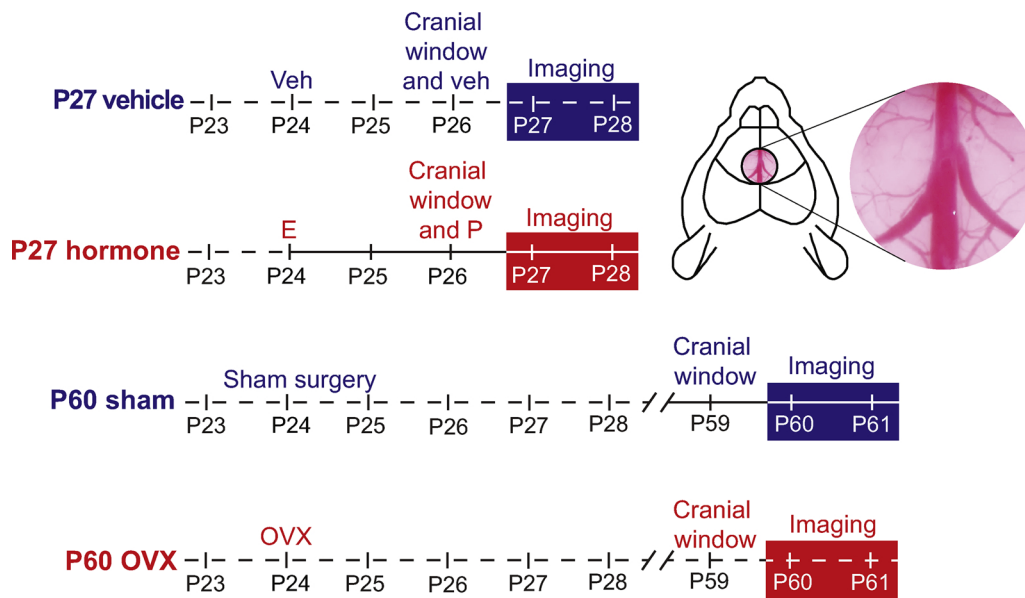
Spine pruning and stabilization during adolescence may be associated with a reduction in the capacity for flexible learning and reorganization of neural connectivity. The best evidence for this comes from adolescent zebra finches, in which greater baseline spine turnover predicts greater capacity for flexible song learning (Roberts et al., 2010). Thus, the stabilization of spines across adolescence may relate critically to developmental shifts in the capacity for plasticity and learning.

Given the links between spine dynamics and learning, it is of great interest to understand the mechanisms driving the pruning and stabilization of dendritic spines during adolescence. We have previously hypothesized that exposure to gonadal steroids during puberty may

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**Fig. 1.** Experimental timeline. To model early puberty, intact female mice were injected with estradiol (E) at P24 and progesterone (P) at P26 ( $N = 12$  mice), while controls were injected with vehicle at both ages ( $N = 12$  mice). To prevent pubertal exposure to gonadal hormones, female mice were ovariectomized (OVX) or received sham surgery at P24 ( $N = 8$  OVX mice;  $N = 8$  sham mice). On the timelines, solid lines indicate exposure to pubertal gonadal hormones, while dashed lines indicate pre-pubertal status or absence of gonads. A cranial window was placed over the dorsomedial frontal cortex anterior to bregma as shown in the schematic. The inset is a photograph of a representative window taken at the end of cranial window surgery. The diameter of the craniotomy is 2.5 mm. Apical dendrites of layer 5 pyramidal cells were imaged in layer 1 of dorsomedial frontal cortex at P27–P28 in vehicle- and hormone-treated mice and in young adulthood (age range P55–65, labeled as P60) in OVX and sham mice.

decrease capacity for plasticity and circuit remodeling in frontal cortex (Piekarski et al., 2017b), which may be mediated, in part, by reduced spine turnover. Correlational evidence in humans and mice supports this possibility: The onset of spine pruning in human frontal cortex coincides with the average age at puberty onset (Petanjek et al., 2011), and measures of cortical thinning in human structural MRI data correlate with pubertal development (Herting et al., 2015; Herting et al., 2014; Peper et al., 2009). Spine pruning and stabilization in layer 5 pyramidal neurons in mouse frontal cortex also occur during pubertal development (Johnson et al., 2016a), sex-specific changes in cortical synapse density occur during adolescence in rats (Drzewiecki et al., 2016), and hormone treatment can alter spine density and turnover in cortical pyramidal cells of adult mice (Tan et al., 2012; Wang et al., 2017). Although these data suggest a potential role for pubertal hormones in the maturation of spine dynamics, establishing a causal relationship requires experimental manipulation of gonadal hormone exposure during adolescence. It is therefore still unknown whether pubertal hormones influence the maturation of spine dynamics during adolescence.

The role of pubertal hormones in brain maturation is particularly important in light of recent advancement in the age of puberty onset in girls and boys (Herman-Giddens, 2006) and the negative educational and mental health outcomes associated with early-onset puberty in girls (Graber, 2013). We have previously shown that in female mice, pubertal hormones drive maturation of inhibitory neurotransmission in layer 2/3 of the dorsomedial frontal cortex (Piekarski et al., 2017a), a region implicated in a variety of cognitive and affective behaviors in rodents and humans (Blakemore and Robbins, 2012; Felix-Ortiz et al., 2016; Johnson and Wilbrecht, 2011). However, it is unknown whether pubertal hormones drive other aspects of frontal circuit maturation, such as spine pruning and stabilization, or maturation of average spine morphology. Revealing which aspects of frontal circuit maturation are hormone-dependent and which are not is critical for understanding normative adolescent development and the implications of the advancing age of puberty onset in humans.

Here, we show the results of *in vivo* 2-photon microscopy experiments designed to test the role of pubertal hormones in the maturation of spine density and turnover on layer 5 pyramidal neurons in the female mouse frontal cortex. The experiments were initially designed to answer three questions: 1) Do female mice show spine pruning and stabilization in the dorsomedial frontal cortex across adolescence, as has been shown in males (Johnson et al., 2016a); 2) Does pre-pubertal hormone exposure induce an early reduction in spine density and

turnover; and 3) Are gonadal hormones necessary for the maturation of spine density and turnover across adolescence. We found that females, like males, show spine stabilization and pruning across adolescence on layer 5 pyramidal cells in frontal cortex, but these processes are unaffected by pre-pubertal hormone exposure or gonadectomy. These negative data led us to pursue opportunistic follow-up analyses of the morphology of spines to ask, 4) Does pre-pubertal hormone treatment or gonadectomy alter the maturation of spine morphology across adolescence and/or morphological dynamics over 24 h? In these analyses, we did find subtle but significant effects that suggest ovarian hormones do contribute to the morphological maturation of dendritic spines on layer V neurons and the dynamic remodeling of their morphology during early adulthood.

## 2. Methods

### 2.1. Animals

Female C57BL/6J mice from the Thy-1-YFP-H line (Jackson Laboratory, Bar Harbor, Maine), in which a subset of layer 5 pyramidal cells is fluorescently labeled (Feng et al., 2000; Porrero et al., 2010), were bred in our animal facility. We chose to use females in this study due to public health issues surrounding the advancing age of puberty in girls (Graber, 2013; Herman-Giddens, 2006), and based on previous data showing that pubertal hormones in females drive maturation of inhibitory neurotransmission in the dorsomedial region of frontal cortex imaged in this study (Piekarski et al., 2017a). All mice were weaned on postnatal day (P)21 and housed in groups of 2–3 same-sex siblings on a 12:12hr reverse light:dark cycle (lights on at 10PM). All procedures were approved by the Animal Care and Use Committee of the University of California, Berkeley and conformed to principles enunciated in the NIH Guide for the Care and Use of Laboratory Animals.

### 2.2. Pre-pubertal hormone exposure to induce early-onset puberty

To advance age at puberty onset, gonadally intact females were injected with 17 beta-estradiol benzoate (0.01 mg/kg subcutaneous) at P24 and progesterone (20 mg/kg subcutaneous) at P26 (Fig. 1; (Piekarski et al., 2017a)). This treatment advances first peripubertal exposure to gonadal steroids and is sufficient to induce endogenous puberty (Ramirez and Sawyer, 1965; Smith and Davidson, 1968). A vehicle control group was injected with equivalent volumes of oil

vehicle at P24 and P26.

### 2.3. Ovariectomy

To eliminate gonadal hormone exposure during puberty, ovariectomies were performed before puberty onset (P24 or P25). Mice were injected with 0.05 mg/kg buprenorphine and 10 mg/kg meloxicam subcutaneously before surgery and were anesthetized with 1–2% isoflurane during surgery. The incision area was shaved and scrubbed with ethanol and betadine. Ophthalmic ointment was placed over the eyes to prevent drying. A 1 cm incision was made with a scalpel in the lower abdomen across the midline to access the abdominal cavity. The ovaries were clamped off from the uterine horn with locking forceps and ligated with sterile sutures. After ligation, ovaries were excised with a scalpel. The muscle and skin layers were then sutured, and wound clips were placed over the incision for 7–10 days to allow the incision to heal. An additional injection of 10 mg/kg meloxicam was given 12–24 h after surgery. Sham control surgeries were identical to ovariectomies except that the ovaries were simply visualized and were not clamped, ligated or excised. Mice were allowed to recover on a heating pad until ambulatory and were post-surgically monitored for 7–10 days to check for normal weight gain and signs of discomfort/distress.

### 2.4. Cranial window surgery

Cranial window surgeries were performed in YFP-H mice at P26 or in young adulthood (age range P54–P63). Detailed surgical procedures have been described previously (Holtmaat et al., 2009). Briefly, mice were anesthetized with isoflurane anesthesia. A craniotomy (diameter 2.5 mm) was made over the dorsomedial frontal cortex, centered over the midline, with the caudal edge of the craniotomy at bregma. The dura was left intact during this procedure. The craniotomy was covered with a thin layer of agarose solution (0.7% in ACSF) and sealed with a glass coverslip. Mice were given subcutaneous injections of meloxicam (10 mg/kg) during surgery and 24 h after surgery.

### 2.5. Dendritic spine imaging

After 1 day of recovery from cranial window surgery, mice were imaged once per day for 2 consecutive days. We chose a 24-h interval between imaging sessions based on our previous data showing differences in spine density and turnover between pre- and post-pubertal male mice using this same imaging interval (Johnson et al., 2016a). Imaging took place at P27 and P28 for vehicle- and hormone-treated mice and between P55 and P65 for ovariectomized (OVX) and sham mice. Rapid bone growth from the rostral midline in young animals makes longer recovery times infeasible for imaging of structures close to the midline in frontal cortex. Previous data from our laboratory show no difference in spine turnover depending on the length of recovery time from cranial window surgery in this brain region (Munoz-Cuevas et al., 2013).

Detailed *in vivo* imaging procedures have been described previously (Holtmaat et al., 2009). Briefly, the apical dendrites of YFP-expressing pyramidal cells (layer 5) were imaged using a Mai Tai HP laser (950 nm, Spectra Physics, Santa Clara, CA), Ultima IV *in vivo* laser-scanning microscope (Bruker, Middleton, WI), and a 40 × 0.8N objective (Olympus, Center Valley, PA). Segments of dendrite (each ~40 μm in length) located within 100 μm of the surface (i.e. in layer 1) were imaged at a resolution of 0.08 μm/pixel using a z-step size of 1 μm. Imaged dendrite segments were less than 0.8 mm from the midline in both the right and left hemispheres, anterior to bregma (0–2.5 mm from bregma). Multiple cells were sampled in each mouse.

### 2.6. Image processing and analysis

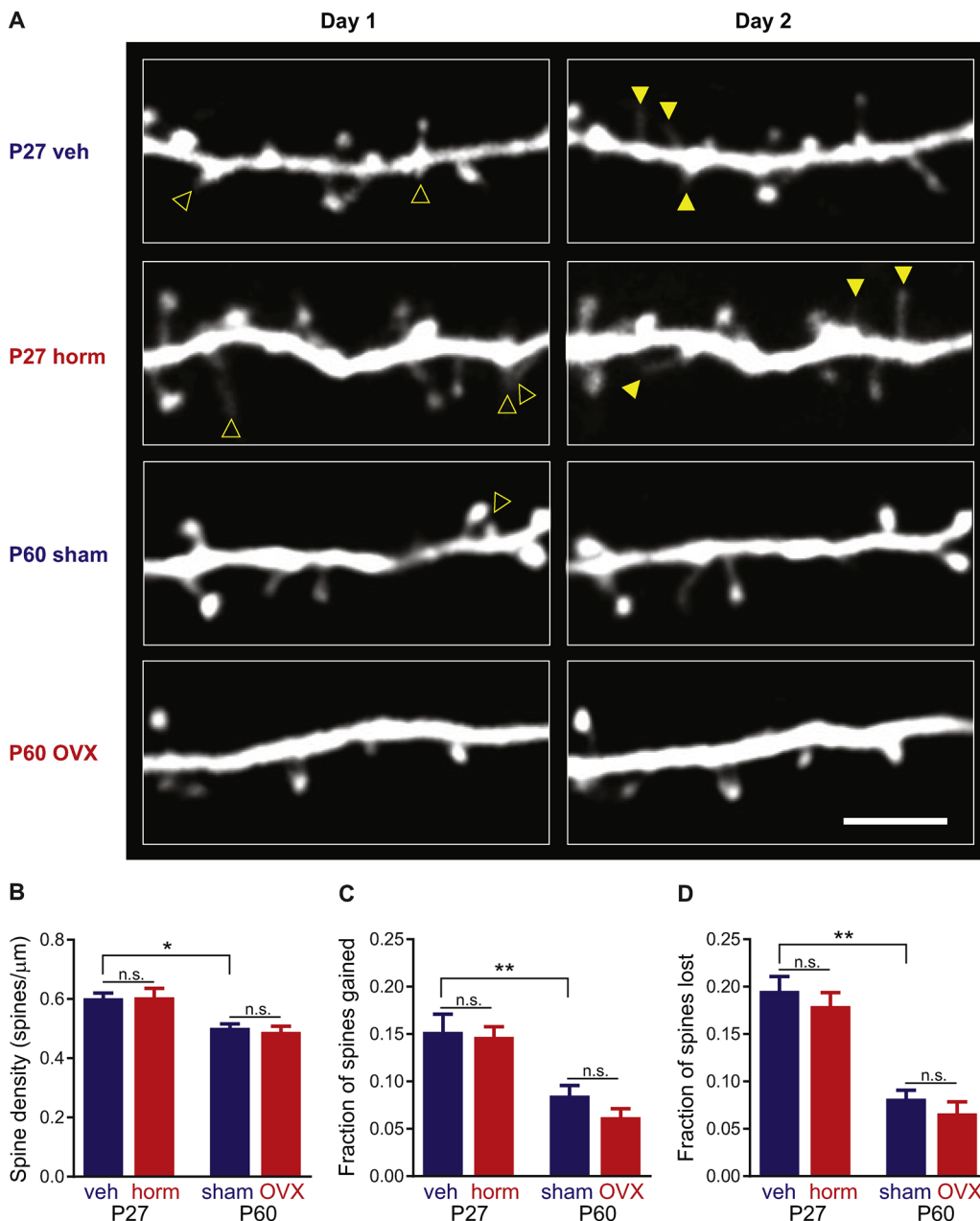
Images were analyzed blind to the mouse's experimental group. Images used for analysis were median-filtered 3-dimensional z stacks. Dendritic spines were scored according to established criteria (Holtmaat et al., 2009) using custom Matlab software (Mathworks, Natick, MA). Dendritic spines were scored if they protruded laterally more than 0.4 μm from the dendritic shaft. To calculate spine density, the total number of spines for each mouse was divided by the total length of analyzed dendrite in μm. To calculate the fraction of spines gained for each mouse, the number of new spines present on the second day of imaging was divided by the total number of spines present on the first day of imaging. To calculate the fraction of spines lost, the number of spines lost between the first and second day of imaging was divided by the total number of spines present on the first day of imaging.

Based on previous studies showing that spine brightness is proportional to spine volume (Holtmaat et al., 2005), we used spine head brightness as a proxy for the relative size of spine heads (Cane et al., 2014). Brightness quantification was performed in ImageJ. A 5 by 5-pixel square (pixel size 0.08 μm) was placed over the spine head, and brightness was calculated as the summed intensity of pixels within the square. To control for differences in fluorophore expression level, spine head brightness was normalized to (i.e. divided by) the brightness of the adjacent dendritic shaft. The brightness of the adjacent shaft was measured as the summed intensity of pixels in a 5 by 5-pixel square placed on the dendritic shaft at the base of each spine. Background brightness, i.e. the summed intensity of pixels in a 5 by 5-pixel square placed over the adjacent background, was subtracted from all shaft and spine measures. Spine length was calculated as the Euclidean distance from the base of the spine to the tip of the spine head.

For image presentation (Fig. 2A), the relevant sections of dendrite were projected onto a 2-dimensional image, which was then Gaussian filtered and contrasted for presentation. To project the relevant sections of dendrite onto a 2-dimensional image, the frames from the 3-dimensional z stack in which spines were most clearly in focus were combined using the maximum or average intensity z projection functions in ImageJ. In one case, this 3D image flattening method caused a failure to display one spine that was scored as present during both imaging sessions but was not in focus in the same frames in which other spines were in focus. All spine gains and losses are faithfully represented and marked by yellow triangles in the z-projected images shown in Fig. 2A.

### 2.7. Statistical tests

We analyzed 132 ± 4.42 (mean ± SEM) spines per mouse. For Fig. 2, we calculated a single value for density, fraction of spines gained, and fraction of spines lost for each mouse, such that N for analysis was the number of mice. For Fig. 3 (analysis of spine brightness and length), spines were considered individually, such that N for analysis was the number of spines. All statistical comparisons were performed using GraphPad Prism (GraphPad, San Diego, CA). Data were tested for normality using a D'Agostino and Pearson omnibus normality test and were tested for equal variance using a Brown-Forsythe test. Data that were normally distributed and had equal variance were analyzed using a 1-way ANOVA with Sidak's corrections for multiple comparisons. Data that were not normally distributed were analyzed using a Kruskal Wallis test with Dunn's corrections for multiple comparisons. For all analyses of group differences (Figs. 2, 3 A–G), 3 post-hoc comparisons were performed: P27 vehicle to P60 sham, P27 vehicle to P27 hormone, and P60 sham to P60 OVX. For analyses of differences between persistent and dynamic spines (Fig. 3H–I), 2 post-hoc comparisons were performed: session 1 persistent vs session 1 lost, and session 2 persistent vs session 2 gained.



**Fig. 2.** Spine density and turnover on layer 5 cells decline with age but are not affected by manipulations of ovarian hormones. (A) Sample images. Open triangles mark spines that were lost between imaging days 1 and 2; filled triangles mark spines that were gained between imaging days 1 and 2. One spine that was not analyzed appears on the right side of the P27 hormone image for day 1; the primary protrusion of this spine was in the z plane. Scale bar represents 5  $\mu\text{m}$ . Spine density (B), spine gains (C), and spine losses (D) were lower in post-pubertal P60 mice compared to pre-pubertal P27 mice. Neither hormone injections nor ovariectomy altered spine density (B), spine gains (C), or spine losses (D). N = 12 P27 vehicle mice; N = 12 P27 hormone mice; N = 8 P60 sham mice; N = 8 P60 OVX mice. All graphs are mean  $\pm$  S.E.M. \*Sidak's adjusted  $p < 0.05$ , \*\*Sidak's or Dunn's adjusted  $p < 0.01$ .

### 3. Results

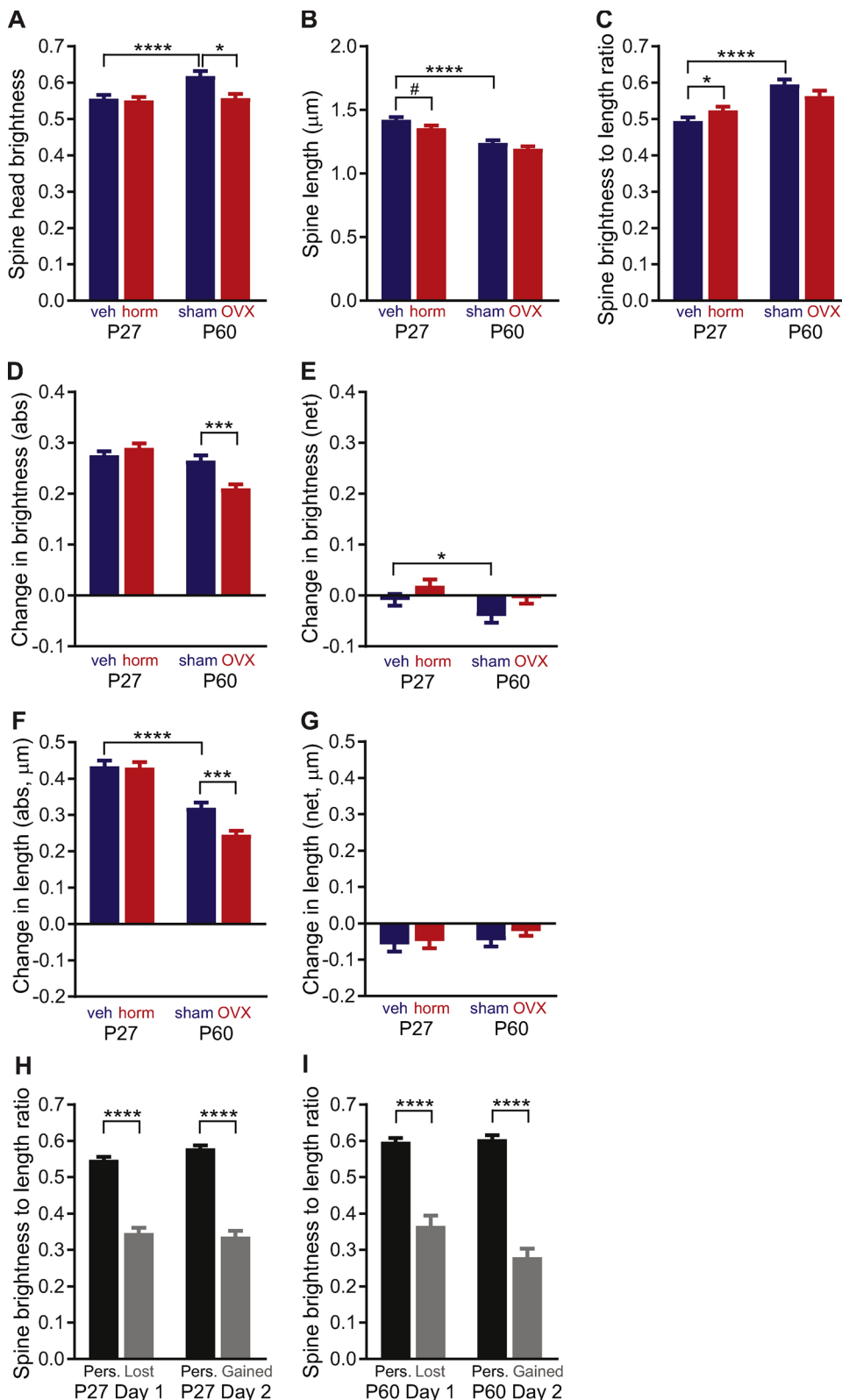
To test whether pubertal hormones drive maturation of spine dynamics across adolescence, we used *in vivo* 2-photon microscopy to follow individual dendrites across two consecutive days in the dorsomedial frontal cortex of female mice. Treatment groups included a vehicle-treated pre-pubertal group (P27 vehicle; Fig. 1), an age-matched hormone-treated group that experienced early-onset puberty (P27 hormone; Fig. 1), a P60 group that went through puberty naturally (P60 sham; Fig. 1), and a P60 group that was ovariectomized before puberty and was therefore not exposed to gonadal hormones during adolescence (P60 OVX; Fig. 1). All imaged mice were from the YFP-H line, which labels a subset of layer 5 pyramidal cells (Feng et al., 2000; Porrero et al., 2010). Imaged dendrites were apical dendrites located in layer 1 of dorsomedial frontal cortex, less than 0.8 mm from the midline in both the right and left hemispheres (Figs. 1, 2 A).

We found that the 4 treatment groups differed in spine density (Fig. 2B;  $F_{3,36} = 6.99$ ,  $p = 0.0008$ ), spine gains (Fig. 2C;  $F_{3,36} = 9.55$ ,

$p < 0.0001$ ), and spine losses (Fig. 2D;  $H = 23.66$ ,  $p < 0.0001$ ). To determine whether female mice show spine pruning and stabilization in dorsomedial frontal cortex across adolescence, we compared P27 vehicle-treated mice to P60 sham mice. We hypothesized that these hormonally unmanipulated female mice would show lower spine density and turnover at P60 compared to P27, as previously observed in male mice (Johnson et al., 2016a). In accordance with our hypothesis, P60 sham mice showed lower spine density (Fig. 2B; Sidak's adjusted  $p = 0.015$ ), lower spine gains (Fig. 2C; Sidak's adjusted  $p = 0.0068$ ), and lower spine losses (Fig. 2D; Dunn's adjusted  $p = 0.0021$ ) compared to P27 vehicle-treated mice.

To test whether early-onset puberty can induce precocious maturation of spine dynamics, we compared P27 vehicle-treated mice to P27 mice that underwent hormone treatment to induce early-onset puberty (Fig. 1; (Piekarski et al., 2017a; Ramirez and Sawyer, 1965; Smith and Davidson, 1968)). We hypothesized that hormone treatment would induce a precocious reduction in spine density and turnover in P27 mice. Contrary to our hypothesis, hormone-treated mice did not





**Fig. 3.** Spine morphology matures across adolescence and is affected by manipulation of pubertal hormones. (A–C) Data shown are from the first day of imaging. N = 1463 spines from P27 vehicle-treated mice; N = 1449 spines from P27 hormone-treated mice; N = 949 spines from P60 sham mice, N = 851 spines from P60 OVX mice. (A) Spine head brightness, used as a proxy for spine head size, increased across adolescence in hormonally unmanipulated mice and was reduced by OVX. All measures of spine head brightness are background-subtracted and normalized to the brightness of the adjacent shaft. (B) Spine length decreased across adolescence in hormonally unmanipulated mice, and hormone treatment showed a trend toward reducing spine length in P27 mice. (C) Spine brightness to length ratio, a measure of spines' morphological maturity, increased across adolescence in hormonally unmanipulated mice, and hormone treatment at P27 hastened this morphological maturation. (D–G) Data shown are for spines that persisted for both imaging days. N = 1173 P27 vehicle spines; N = 1173 P27 hormone spines; N = 870 P60 sham spines, N = 793 P60 OVX spines. (D) OVX reduced day-to-day changes in spine head brightness, when changes were considered as absolute values. (E) Net change in spine head brightness was close to zero for all groups, suggesting that increases in spine head size were largely balanced by decreases, although P60 sham mice showed slightly greater reductions in spine head size compared to P27 vehicle mice. (F) Changes in spine length (absolute values) decreased across adolescence and were further reduced by OVX. (G) Net change in spine length did not differ among the groups. (H–I) Spines that persisted for both imaging days showed greater brightness to length ratios compared to spines that were gained or lost. (H) N = 2346 persistent spines, 566 lost spines, and 434 gained spines. (I) N = 1663 persistent spines, 137 lost spines, and 132 gained spines. All graphs are mean  $\pm$  S.E.M. #Dunn's adjusted  $p = 0.05$ . \*Dunn's adjusted  $p < 0.05$ . \*\*\*Dunn's adjusted  $p < 0.001$ . \*\*\*\*Dunn's adjusted  $p < 0.0001$ .

differ from vehicle-treated mice in spine density (Fig. 2B; Sidak's adjusted  $p > 0.99$ ), gains (Fig. 2C; Sidak's adjusted  $p = 0.99$ ), or losses (Fig. 2A; Dunn's adjusted  $p > 0.99$ ).

To determine whether gonadal hormone exposure during puberty was necessary for the adolescent reduction in spine density and turnover, we compared mice that were ovariectomized before puberty to gonadally intact sham controls that were imaged post-pubertally. We

hypothesized that pre-pubertal gonadectomy would prevent the adolescent decrease in spine density and turnover. Contrary to our hypothesis, P60 OVX mice did not differ from P60 sham mice in spine density (Fig. 2B; Sidak's adjusted  $p = 0.98$ ), gains (Fig. 2C; Sidak's adjusted  $p = 0.68$ ), or losses (Fig. 2D; Dunn's adjusted  $p > 0.99$ ). These results indicate that spine pruning and stabilization occur across adolescence in the dorsomedial frontal cortex of female mice, and that

in the layer 5 neurons investigated here, these processes do not depend on gonadal hormone exposure during puberty.

To determine whether spine morphologies differed based on age and hormonal status, we quantified the length and spine head brightness of all analyzed spines. Spine head brightness was used as a proxy for spine head size based on previous data showing that brightness correlates with spine volume (Cane et al., 2014; Holtmaat et al., 2005). Spine head brightness differed among the groups (Fig. 3A;  $H = 26.32$ ,  $p < 0.0001$ ). P60 sham mice showed brighter, i.e. larger, spine heads compared to P27 vehicle-treated mice (Dunn's corrected  $p < 0.0001$ ), suggesting that spine head size increases across adolescence in mice undergoing natural puberty. Hormone treatment did not alter spine head brightness in P27 mice (Dunn's adjusted  $p > 0.99$ ), but P60 OVX mice showed dimmer spine heads compared to P60 sham mice (Dunn's adjusted  $p = 0.0498$ ), suggesting that pubertal hormones contribute to the increase in spine head size across adolescence. Spine length decreased across adolescence in hormonally unmanipulated mice (Fig. 3B;  $H = 38.74$ ,  $p < 0.0001$ ; Dunn's adjusted  $p < 0.0001$  for P27 vehicle vs P60 sham). P27 hormone-treated mice showed a trend toward shorter spine length compared to P27 vehicle-treated mice (Dunn's adjusted  $p = 0.05$ ), but spine length did not differ between P60 OVX and P60 sham mice (Dunn's adjusted  $p = 0.89$ ). Spine head brightness (i.e. size) to spine length ratio, a measure of spines' morphological maturity (reviewed in (Berry and Nedivi, 2017)), differed among the groups (Fig. 3C;  $H = 51.4$ ,  $p < 0.0001$ ). P60 sham mice showed greater brightness to length ratio compared to P27 vehicle-treated mice (Dunn's adjusted  $p < 0.0001$ ), suggesting that spines develop more mature morphologies across adolescence in mice undergoing natural puberty. Hormone treatment subtly, but significantly, increased brightness to length ratio in P27 mice (Dunn's adjusted  $p = 0.04$ ), while a comparison of P60 sham to P60 OVX mice did not reach significance after correcting for multiple comparisons (Dunn's adjusted  $p = 0.15$ ). These results suggest that 1) spine morphology matures across adolescence, with spine head brightness (i.e. size) increasing and spine length decreasing, and 2) that gonadal hormones may contribute to this morphological maturation.

For spines that persisted across both imaging days, we measured the change in spine head brightness and spine length from day 1 to day 2. Changes in brightness and length were considered as absolute values, in which both growth and retraction events were counted as positive numbers (Fig. 3D, F), and as net change, in which growth events were counted as positive and retraction events as negative (Fig. 3E, G). Absolute change in spine head brightness differed among the groups (Fig. 3D;  $H = 45.69$ ,  $p < 0.0001$ ). A comparison of P27 vehicle to P60 sham mice did not reach significance (Dunn's adjusted  $p = 0.21$ ), but P60 OVX mice showed less change in spine head brightness compared to P60 sham mice (Dunn's adjusted  $p = 0.0009$ ), suggesting that gonadal hormones may support day-to-day changes in spine head size in adult mice. Net change in spine head brightness was close to zero in all groups, suggesting that spine head growth was largely balanced by spine head shrinkage, but differed slightly between P27 vehicle and P60 sham animals (Fig. 3E;  $H = 13.09$ ,  $p = 0.0045$ ; Dunn's adjusted  $p = 0.0495$  for P27 vehicle vs P60 sham). Absolute change in spine length also differed among the groups (Fig. 3F;  $H = 127.6$ ,  $p < 0.0001$ ). P60 sham animals showed less change in spine length compared to P27 vehicle-treated animals (Dunn's adjusted  $p < 0.0001$ ), suggesting that spine growth and retraction events decrease across adolescence in mice undergoing natural puberty. However, P60 OVX animals showed less change in spine length compared to P60 sham animals (Dunn's adjusted  $p = 0.0007$ ), suggesting that gonadal hormones in adulthood may support day-to-day changes in spine length. Net change in spine length did not differ among the groups (Fig. 3G;  $H = 2.14$ ,  $p = 0.54$ ).

When persistent spines were compared to those that were gained or lost, persistent spines showed dramatically greater brightness to length ratios, i.e. more mature spine morphology, compared to spines that

were gained or lost (Fig. 3H–I;  $H = 416.8$ ,  $p < 0.0001$  for P27 mice;  $H = 169.7$ ,  $p < 0.0001$  for P60 mice; Dunn's adjusted  $p < 0.0001$  for all comparisons of persistent to gained or lost spines). This result indicates that spine turnover events were dominated by spines with immature morphologies in both age groups (Fig. 3H–I), despite the fact that P60 spines on average showed more mature morphologies than P27 spines (Fig. 3A–C).

#### 4. Discussion

Our results indicate that, as we might predict from males, female mice show pruning and stabilization of spines on layer 5 pyramidal cells in the dorsomedial frontal cortex, as indicated by greater spine density, gains, and losses in pre-pubertal P27 mice compared to post-pubertal P60 mice (Fig. 2B–D). Our manipulations of ovarian hormones suggest that gonadal hormones during puberty do not regulate these processes of spine pruning and stabilization of spine turnover: Pre-pubertal gonadal hormone exposure did not accelerate spine pruning and stabilization in P27 mice, and pre-pubertal gonadectomy did not alter spine density or turnover in mice imaged at P60 (Fig. 2B–D). However, our exploratory analyses of spine morphology suggest that pubertal hormones may contribute to the maturation of spine morphology across adolescence, with pre-pubertal OVX reducing spine head brightness (i.e. size) in adulthood (Fig. 3A) and pre-pubertal hormone treatment driving a slight increase in measures of morphological maturity in P27 mice (Fig. 3B–C). Furthermore, pre-pubertal OVX reduced day-to-day changes in both spine head brightness and spine length (Fig. 3D, F), suggesting that gonadal hormones may support morphological remodeling of persistent spines in adult female mice. Thus, although gonadal hormones do not drive the reduction in frontal cortex spine density and turnover across adolescence, gonadal hormones may alter spine morphology in subtler ways during both adolescence and adulthood.

Dendritic spines are critical for the integration of excitatory and inhibitory inputs on cortical pyramidal cells (Spruston, 2008), and their stabilization across adolescence may relate to changes in the capacity for experience-dependent circuit remodeling and flexible learning (Roberts et al., 2010) and/or the consolidation of learned associations (Johnson et al., 2016a; Johnson et al., 2016b; Lai et al., 2012; Munoz-Cuevas et al., 2013). The process of spine stabilization across adolescence may therefore be fundamental to the maturation of circuit dynamics and behavior, and our results suggest that this process can proceed on layer 5 pyramidal cells in the absence of pubertal exposure to gonadal hormones.

Although the lack of hormone effects on spine density and turnover in our data is unambiguous, one limitation of this experiment is that we sampled from only two timepoints: one pre-pubertal age (P27) and one post-pubertal age (P60). For hormone- and vehicle-treated mice, we chose to assay spine density and turnover at P27–28 based on our previous results showing that the same hormone treatment protocol alters inhibitory neurotransmission in L2/3 of frontal cortex at P27–28 (Piekarski et al., 2017a). However, it is possible that pre-pubertal hormone exposure may influence the maturation of spine density and turnover over a more protracted time course, with effects that accumulate to a detectable level at later ages. Future studies may include hormone- and vehicle-treated groups sampled at older ages. Similarly, while we did not observe effects of OVX in mice imaged at P60, it is possible that OVX altered the timing or pace of spine pruning and stabilization across adolescence, but that the OVX and sham groups converged by P60. To test this possibility, future studies may include OVX and sham groups imaged during adolescence.

A second limitation of the present study is that in the YFP-H mouse line we used for spine imaging, only a subset of layer 5 neurons is labeled (Feng et al., 2000; Porrero et al., 2010). While this sparse labeling is necessary for following individual spines from one day to the next, it is possible that pubertal hormones alter spine dynamics in cell types we

did not image. In layer 5 of the frontal cortex, pyramidal cells can be separated into those that project to the pyramidal tract (PT-type) and those that project elsewhere within the cortex (intra-telencephalic projecting, i.e. IT-type) (Porrero et al., 2010). In YFP-H mice, YFP-expressing cells are primarily PT-type (Porrero et al., 2010), and less is known about the adolescent maturation of IT-type cells. However, there are data suggesting that YFP-negative, IT-type neurons mature differently than YFP-positive, PT-type neurons. In YFP-H mice, miniature excitatory and inhibitory currents (mPSCs) are stable from P25 to P40 on YFP-expressing cells, while neighboring YFP-negative (IT-type) cells show an increase in miniature inhibitory current (mIPSC) amplitude (Vandenberg et al., 2015). Maturation of mIPSC amplitude onto these YFP-negative layer 5 cells is dependent on TrkB signaling (Vandenberg et al., 2015). TrkB signaling, in turn, could be regulated by gonadal hormone effects on brain derived neurotrophic factor (BDNF) in both male and female rodents (Carbone and Handa, 2013). Thus, although gonadal hormones do not regulate the pruning and stabilization of spines on the YFP-expressing (PT-type) neurons assayed in our current study, future work in IT-type layer 5 cells may yet reveal gonadal hormone effects on the adolescent pruning and stabilization of spines.

Layer 2/3 pyramidal neurons may also differ from layer 5 subtypes and merit independent investigation. Cells in layer 2/3 and layer 5 of the cortex are generated at different times (Frantz and McConnell, 1996; Rakic, 1988), and likely also develop differently. We have recently found that gonadal hormones are critical for an increase in miniature inhibitory current frequency and an increase in probability of GABA release onto layer 2/3 neurons in frontal cortex during puberty (with no effects on current amplitude), and prepubertal hormone injections also decrease tonic inhibitory current in these same layer 2/3 cells (Piekariski et al., 2017a). In follow-up experiments, it will be critical to test if hormonal manipulation alters the density and turnover of dendritic spines in layer 2/3 neurons and layer 5 IT-type neurons, both of which are harder to access using standard Thy-1 based *in vivo* imaging methods.

If hormones do not play a role in the pruning and stabilization of dendritic spines on layer 5 neurons, then what factors drive these processes? Age, neural activity, and stress hormones can be linked to changes in intracellular proteins (Gray et al., 2006; Hamilton et al., 2017; Liston et al., 2013) and cell–cell adhesion molecules (Kwon et al., 2012) that may play a regulatory role in spine maturation. Experience itself (or the lack of experience) may also drive the stabilization of synapses and serve to regulate plasticity (Johnson et al., 2016b; Panchanathan and Frankenhuis, 2016; Roberts et al., 2010; Wilbrecht et al., 2010; Wilbrecht et al., 2006; Zuo et al., 2005b).

Collectively, the present layer 5 spine data and previously published data on inhibitory neurotransmission onto layer 2/3 pyramidal cells (Piekariski et al., 2017a) illustrate that some aspects of frontal circuit maturation rely on gonadal hormones, while others do not. Thus, individual differences in the timing of puberty could result in differences in the relative timing of hormone-dependent and hormone-independent maturational processes. In recent decades, the average age at puberty onset has advanced, particularly in girls, and early-onset puberty is associated with negative mental health outcomes in girls (Herman-Giddens, 2006). We speculate that differences in the relative timing of maturation of different layers or circuits in the frontal cortex could play a role in the negative mental health outcomes associated with early-onset puberty in girls (Graber, 2013). It is also possible that extrinsic manipulation of pubertal hormones through contraceptive use and gender-affirming hormone treatment may have neurodevelopmental implications that should be clarified through further research.

## 5. Conclusions

The pruning and stabilization of dendritic spines across adolescence occur in a variety of cortical regions and may relate to shifts in the capacity for experience-dependent circuit remodeling and flexible

learning. Here, we show that on a subset of layer 5 pyramidal cells in the dorsomedial frontal cortex, the pruning and stabilization of spines measured at two timepoints occur in a gonadal hormone-independent manner. This work contrasts with our recent studies showing that pubertal hormones drive maturation of inhibitory neurotransmission in layer 2/3 of this same brain region (Piekariski et al., 2017a). Our results suggest that multiple maturational processes occur in the frontal cortex during adolescence and are independently controlled via distinct mechanisms, with some systems sensitive to gonadal hormone exposure and others maturing in a gonadal hormone-independent manner. The timing of pubertal hormone exposure may therefore alter the sequence or phase relationships among these maturational processes, indicating a need for further research on the neurodevelopmental implications of the advancing age of puberty onset and the use of gonadal hormone treatments in youth.

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## Conflict of interest

None.

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